

Pulsatile Atheroprone Shear Stress Affects the Expression of Transient Receptor Potential Channels in Human Endothelial Cells

Florian Thilo, Bernd J. Vorderwülbecke, Alex Marki, Katharina Krueger, Ying Liu, Daniel Baumunk, Andreas Zakrzewicz, Martin Tepel

Abstract—The goal of the study was to assess whether pulsatile atheroprone shear stress modulates the expression of transient receptor potential (TRP) channels, TRPC3, TRPC6, TRPM7, and TRPV1 mRNA, in human umbilical vascular endothelial cells. Exposure of cultured vascular endothelial cells to defined shear stress, producing a constant laminar flow (generating a shear stress of 6 dyne/cm²), laminar pulsatile atheroprotective flow (with a mean shear stress of 20 dyne/cm²), or laminar atheroprone bidirectional flow (with a mean shear stress of 0 dyne/cm²) differentially induced TRPC6 and TRPV1 mRNA as measured by quantitative real-time RT-PCR and normalized to GAPDH expression. Thereby, TRPC6 and TRPV1 mRNA expressions were significantly increased after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile. Furthermore, the expression of transcription factors GATA1 and GATA4 was significantly correlated with the expression of TRPC6 mRNA. In contrast, after 24 hours of constant laminar flow, the expression of TRPC6 and TRPV1 mRNA was unchanged, whereas the expression of TRPC3 and TRPM7 was significantly higher in endothelial cells exposed to shear stress in comparison with endothelial cells grown under static conditions. There was a significant association between the expression of TRPC6 and tumor necrosis factor- α mRNA in human vascular tissue. No-flow and atheroprone flow conditions are equally characterized by an increase in the expression of tumor necrosis factor- α ; however, inflammation-associated endothelial cell reactions may be further aggravated at atheroprone flow conditions by the increase of TRPV1 and TRPC6, as observed in our study. (*Hypertension*. 2012;59:1232-1240.)

Key Words: TRP channels ■ mRNA expression ■ shear stress ■ endothelial cells

Atherosclerosis correlates with regional hemodynamics. Atherosclerotic lesions are more likely to occur at specific sites, for example, bifurcations, in the arterial system. These sites are characterized by altered shear stress, specifically by atherogenic flow patterns composed of low flow, flow separation, relatively steep shear stress gradients, reversal of flow, and turbulent flow.¹ Shear stress, defined as the tensile force tangentially acting on endothelial cells, is elicited by the flowing blood.² It has been shown that the force and direction of shear stress are essential for atherosclerotic lesions to occur and are, thus, linked to cardiovascular disease and susceptibility for atherosclerosis in particular. So, shear stress of low magnitude and reversing direction changes the endothelial cell's phenotype toward a chronic inflammatory reaction in the preatherosclerotic endothelium.³ Accordingly, atherosclerosis-susceptible and atherosclerosis-resistant regions of the human carotid arteries

are characterized by different pulsatile profiles of wall shear stress. These different profiles are represented by an atheroprone prototypic arterial waveform versus an atheroprotective prototypic arterial waveform, which can be replicated in vitro.^{4,5}

An early reaction of endothelial cells toward shear stress is the activation of flow-sensitive ion channels, proposed to act as flow sensors.⁶ Moreover, several subtypes of transient receptor potential (TRP) channels, including transient receptor potential cation channel, subfamily C, member 3 (TRPC3) TRPC6, TRPM7, and TRPV1, have been linked to cardiovascular disease, hypertension, and atherosclerosis.^{7–13} TRP channels are activated by a variety of factors, including chemical stimuli and mechanical stimuli, like shear stress. TRP channels are known to be mechanosensitive, although the specific mechanisms still need to be elucidated.⁷ In addition, alterations in blood flow are well known to have an

Received September 19, 2011; first decision October 18, 2011; revision accepted April 10, 2012.

From the Department of Nephrology (F.T., K.K.) and Shear Stress and Vascular Biology Research Group, Institute of Physiology (B.J.V., A.M., A.Z.), Charité Campus Benjamin Franklin, Berlin, Germany; Institute of Molecular Medicine, Cardiovascular and Renal Research (Y.L., M.T.), Institute of Clinical Research, Odense University Hospital and University of Southern Denmark, Odense, Denmark; Department of Urology (Y.L.), Tenth People's Hospital, Tongji University, Shanghai, China; Department of Urology (D.B.), Otto-von-Guericke University, Magdeburg, Germany.

A.Z. and M.T. contributed equally to this work.

Correspondence to Florian Thilo, Department of Nephrology, Charité Campus Benjamin Franklin, Thielallee 71, 14195 Berlin, Germany. E-mail Florian.Thilo@charite.de

© 2012 American Heart Association, Inc.

Hypertension is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.111.183608

effect on the expression of many genes, which harbor promoter regions responsive to shear stress.¹ With respect to TRPCs, different flow types could, thus, change endothelial TRPC expression, which, in turn, might change the responsiveness of endothelial cells to flow. In the present study we, therefore, tested the hypothesis that shear stress produced from pulsatile atheroprone, on one hand, or pulsatile atheroprotective flow, on the other, differentially affects the expression of TRP channel transcripts in human endothelial cells. We, thus, investigated changes of TRPC3, TRPC6, TRPM7, and TRPV1 mRNA expression in human umbilical vascular endothelial cells (HUVECs) exposed to either atheroprotective or atheroprone pulsatile profiles of shear stress.

Experimental Procedures

Cell Culture

HUVECs were isolated and cultivated as described previously¹⁴ using Endothelial Cell Basal Medium MV1 with Cell Growth Supplement Pack MV1 (PromoCell, Heidelberg, Germany) and used at passage one. All of the cells were exposed to shear stress 1 day after confluence.

Shear Stress Experiments

Shear stress was applied to cell monolayers using a cone-and-plate system for 24 hours, except for the experiments with LY-294002, where a duration time of 4 hours was used, as described previously.¹⁵ Pulsatile atheroprotective or atheroprone flow profiles were taken from the literature.⁴ If a set of shear stress experiments involved values higher than 6 dyne/cm² (1 dyne=100 mN), the viscosity of the medium of these samples and the corresponding control samples was increased to 5 cP with dextran (molecular weight, 100 000–200 000 g/mol; Sigma-Aldrich, Taufkirchen, Germany). Control samples were from the same isolation and cultivated under nonflow conditions for the same time as their noncontrol counterparts.

Inhibition of phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI3K) was inhibited using LY-294002 (Sigma-Aldrich). A stock solution (10 mmol/L) was prepared in dimethylsulfoxide and used at a final concentration of 10 μ mol/L. HUVECs were preincubated for 30 minutes and then exposed to shear stress.

Preparation of Human Vascular Tissue

Small pieces of human vascular tissue were obtained during the kidney transplant procedure and immediately frozen in RNAlater. One microliter of TRIzol reagent was added to 50 mg of tissue and tissue crushed on ice using an ultraturax instrument for 30 seconds. After vortexing for 3 minutes and centrifugation for 5 minutes at 8000g, the remaining supernatant was transferred to a new tube. All of the patients gave written informed consent, and the study was approved by the local ethics committee.

RNA Isolation and Reverse Transcription

Total RNA was isolated from HUVECs and vascular tissue using the RNeasy mini kit including RNase-free DNase set (Qiagen, Hilden, Germany). Using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany), cDNA was synthesized from 2 μ g of total RNA using oligo dT^{12–18} and 5 U of Avian Myeloblastosis Virus reverse transcriptase at 50°C for 60 minutes, followed by heating to 85°C for 5 minutes.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR for transient receptor potential cation channel subfamily C member 3 (TRPC3), member 6 (TRPC6), subfamily M member 7 (TRPM7), subfamily V member 1 (TRPV1), tumor necrosis factor- α (TNF- α), GATA binding protein 1 (globin

transcription factor 1; GATA1), GATA binding protein 4 (GATA4), and GAPDH was performed using the following primers: TRPC3 (Reference Sequence database accession No. NM_003305.2), forward, 5'-GACTTTGGGATGCTGTCCAT3' and reverse, 5'-GTACGCAATC CGAGAGAAGC3'; TRPC6 (NM_004621.5), forward, 5'-GCCAATG AGCATCTGGAAAT3' and reverse, 5'-TGGAGTCACATCATGGGA GA3; TRPM7 (NM_017672.4), forward, 5'-CTTATGAAGAGGCAG GTCATGG3' and reverse, 5'-CATCTGTCTGAAGGACTG3'; TRPV1 (NM_080704.3, NM_018727.5, NM_080705.3, NM_080706.3), forward, 5'-CAACAAGATCGCACAGGAGA3' and reverse, 5'-TCCT TGCCATCAGGTGTGTA3'; GATA1 (NM_002049.3), forward, 5'-TGGAGACTTTGAAGACAGAGCGGCTGAG3' and reverse, 5'-GAAGCTTGGGAGAGGAATAGGCTGCTGA3'; TNF- α (NM_000594.2), forward, 5'-CCCAGGGACCTCTCTCTAATC3' and reverse, 5'-ATGGGCTACAGGCTTGTCAC3'; GATA4 (NM_008092.3), forward, 5'-AATGCCTGTGGCCTCTATCA3' and reverse, 5'-CTGGT TTGAATCCCCCTCCTT3'; and GAPDH (NM_002046.3), forward, 5'-AACTGCTTAGCACCCCTGGC3' and reverse, 5'-ATGACCTTGCCACAGCCTT3'.

Quantitative RT-PCR was performed in a Real-Time Cycler (Rotor-Gene 2000, Corbett Research) using 2 μ g of RNA, which was added to a final volume of 20 μ L, which contained 10 μ L of 2 \times QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden) and 500 nmol/L of each primer. The reaction was initiated at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing for 30 seconds at 54°C (GAPDH), at 57°C (TRPC6), or at 60°C (TRPC3, TRPM7, TRPV1, TNF- α , GATA1, and GATA4) and extension at 72°C for 15 seconds. After amplification, a melting curve analysis from 50°C to 95°C with a heating rate of 0.2°C per second with continuous fluorescence acquisition was performed to assure correct PCR amplification. Data were recorded on a Real-Time Cycler (Rotor-Gene 2000), and cycle threshold values (crossing points) for each reaction were determined using the Rotor-Gene program (Corbett Research). The relative quantification method was used whereby the change in expression of the target genes (TRPC3, TRPC6, TRPM7, TRPV1, TNF- α , GATA1, and GATA4) relative to the housekeeping gene (GAPDH) was calculated.

Immunoblotting

HUVECs were harvested and washed with PBS. Afterward, cells were centrifuged at 4°C for 3 minutes at 3000 rpm and the supernatant discarded. Then, 300 μ L of lysis buffer containing 25 mmol/L of Tris-HCl (pH 8.0), 1 mol/L of NaCl, 200 mmol/L of EDTA, 250 mmol/L of Octyl- β -D-glucopyranoside, 1 mol/L of sodium fluoride, and complete mini protease inhibitor mixture (Roche Diagnostics) were added. The homogenate was incubated for 30 minutes on ice. The proteins in the supernatant were concentrated using a centrifugal filter device (Amicon ultra; Millipore), mixed with loading buffer, heated up to 100°C for 3 minutes, separated using a denaturing 10% SDS-PAGE at 150 V for 90 minutes, and transferred to pure nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories) at 14 V overnight. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany) for 1 hour at room temperature. Membranes were incubated with primary rabbit antihuman TRPC6 antibody, rabbit antihuman TRPV1 antibody (both Alomone Laboratories, Jerusalem, Israel), or goat antihuman GAPDH antibody (Santa Cruz Biotechnology) at a 1:1000 solution containing 0.1% Tween 20, Odyssey blocking buffer, and PBS for 1 hour, washed 4 times for 5 minutes. Membranes were incubated with the secondary antibody (either IRDye 800 infrared fluorescent dye-conjugated sheep anti-rabbit antibody [Biomol, Hamburg, Germany] or Alexa Fluor 680 infrared fluorescent dye-conjugated donkey anti-goat antibody [Molecular Probes, Eugene, OR]), both at a 1:1000 solution containing 0.1% Tween 20, Odyssey blocking buffer, and PBS for 1 hour and washed 4 times for 5 minutes. Imaging was performed at a wavelength of 700 nm (GAPDH) and 800 nm (TRPC6 and TRPV1).

Patients

We analyzed the expression of TRPC6 and TNF- α mRNA in human vascular tissue from 6 chronic kidney disease patients.

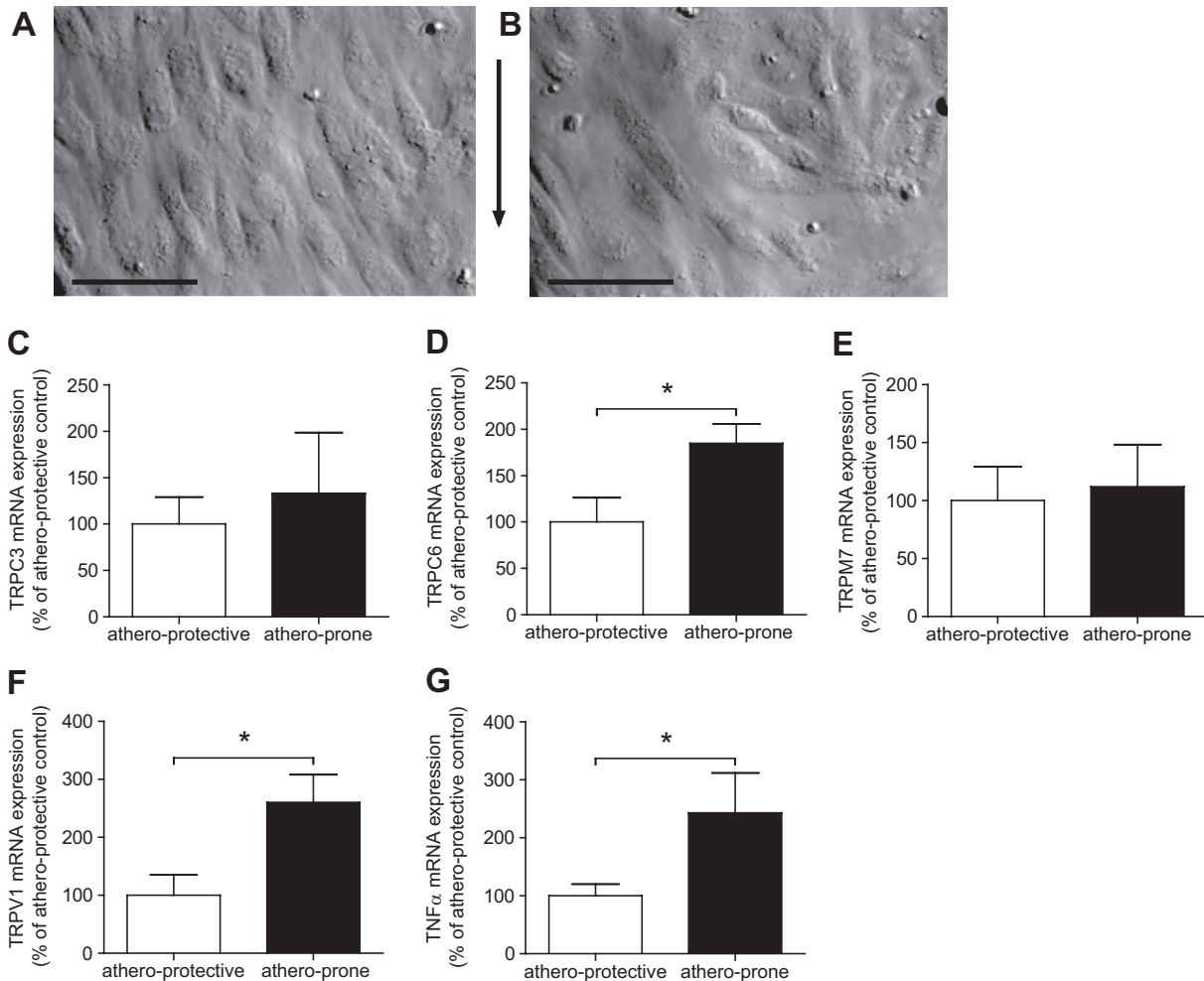


Figure 1. Morphology of human umbilical vascular endothelial cells (HUVECs) after flow exposure (A and B). HUVECs with medium viscosity increased to 5 cP by the addition of dextran were exposed to an atheroprotective (A) or an atheroprone (B) flow profile for 24 hours. There was a significant shear stress-induced HUVEC alignment in response to atheroprotective vs atheroprone conditions. Scale bars indicate 100 μ m. **Arrow** indicates the direction of shear stress. Data show expression of transient receptor potential (TRP) channels in HUVECs after flow exposure (C through G). HUVECs with medium viscosity increased to 5 cP were exposed to an atheroprone or atheroprotective flow profile for 24 hours, and expression of (C) TRPC3, (D) TRPC6, (E) TRPM7, (F) TRPV1, and (G) tumor necrosis factor (TNF)- α transcripts were analyzed by real-time quantitative RT-PCR. * P <0.05 between groups.

Bioinformatics

The core promoter region 2 kb upstream of the reported transcription start site of TRPC6 was analyzed in silico for transcription factor binding sites using the Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess/tess). The analysis was limited to the transcription factors GATA1 (accession No. TRANSFAC Database T00306) and GATA4 (T02687).

Statistical Analysis

All of the data were expressed as mean \pm SEM and were compared using the nonparametric Mann-Whitney test. Relations between variables were investigated using the Spearman correlation. Two-sided P values <0.05 were considered to indicate statistical significance. Where error bars do not appear on the figure, error was within the symbol size. All of the data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA).

Results

Detection of TRP Channel mRNA Using Quantitative RT-PCR

We analyzed the expression of TRPC3, TRPC6, TRPM7, TRPV1, TNF- α , GATA1, GATA4, and GAPDH transcripts

in HUVECs using Quantitative RT-PCR. All of the mentioned types of mRNA species could be detected in HUVECs. The specificity of the PCR was demonstrated by the presence of a single peak in the melting curve analysis.

Atheroprone Shear Stress Increases TRPC6 and TRPV1 mRNA

Morphological changes were analyzed according to the type of shear stress applied. HUVECs exposed to atheroprotective shear stress were more elongated than under atheroprone shear stress. HUVECs exposed to atheroprone shear stress were not aligned but were polygonal and randomly oriented (Figure 1A and 1B). We analyzed the expression of TRP channel mRNA in an established in vitro dynamic flow system,⁴ in which HUVECs are exposed to 2 well-defined types of mechanical stimulation, considered to have direct pathophysiological relevance to atherosclerotic disease in vivo. Specifically, we compared the effect of an atheroprotective flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm², and an

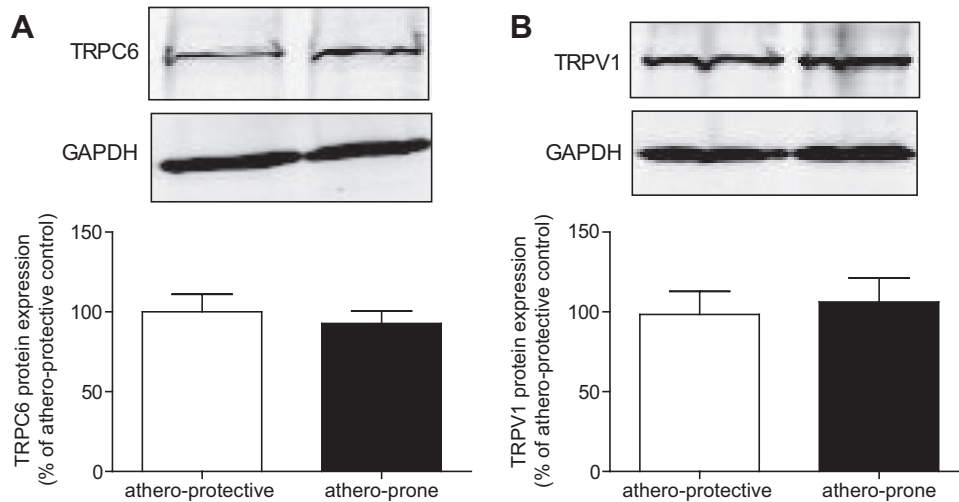


Figure 2. Expression of transient receptor potential (TRP) channels in human umbilical vascular endothelial cells (HUVECs) after flow exposure. HUVECs with medium viscosity increased to 5 cP were exposed to an atheroprone or atheroprotective flow profile for 24 hours, and expressions of (A) TRPC6 and (B) TRPV1 were analyzed by Western blotting. Protein levels were not significantly affected within 24 hours, indicating that only long-term exposure to atheroprone shear stress may cause receptor protein modifications.

atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm^{24,16} on TRP channel mRNA expression. TRPC6 and TRPV1 mRNA expressions were significantly increased after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile (normalized ratio, 185±21 versus 100±26 arbitrary units; $P=0.04$; each $n=6$ for TRPC6; normalized ratio, 260±48 versus 100±35 arbitrary units; $P=0.02$; each $n=6$ for TRPV1). The inflammatory properties of the atheroprone flow profile were well underlined by the significant higher expression of TNF- α mRNA compared with the atheroprotective flow profile (normalized ratio, 243±69 versus 100±20 arbitrary units; $P=0.04$; each $n=6$). There was a significant correlation between the expression of TNF- α and TRPC6 mRNA ($r=0.58$; $P<0.05$), whereas there was no correlation between TNF- α and TRPV1 mRNA (Figure 1C through 1G).

In addition, we compared the effect of an atheroprotective flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm² and an atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm on TRPC6 and TRPV1 channel protein expression. TRPC6 and TRPV1 protein expressions were not changed after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile (normalized ratio, 93±8 versus 100±11 arbitrary units; $P=1.00$; each $n=5$ for TRPC6; normalized ratio, 98±14 versus 106±15 arbitrary units; $P=0.69$; each $n=5$ for TRPV1; Figure 2).

Transcription factors of the GATA family have been validated experimentally as relevant transcription enhancers for TRPC6.¹⁷ In the present study, the expression of GATA1 and GATA4 mRNA was significantly correlated with the expression of TRPC6 mRNA ($P=0.02$ for GATA1; $P=0.04$ for GATA4; Figure 3).

Regular Laminar Shear Stress Increases TRPC3 and TRPM7 mRNA Expression in Endothelial Cells

Morphological changes were analyzed according to the type of shear stress applied. The static conditions displayed a

confluent monolayer with polygonal, randomly oriented, and cobblestone-like HUVECs. Shear stress caused HUVECs to develop leading edges, retract rear edges, become elongated and spindle like, and align parallel in the direction of flow (Figure 4A and 4B).

In contrast to atheroprone shear stress, regular laminar shear stress of ≤ 6 dyne/cm² was chosen, because it can be present at every part of the vascular tree and represents the typical average level in human blood vessels well between venous (≈ 1 dyne/cm²) and arterial conditions (≈ 10 dyne/cm²). As shown in Figure 4, after 24 hours of laminar shear stress, the expression of TRPC6 or TRPV1 mRNA was not significantly changed compared with control conditions (68±25 versus 100±51 arbitrary units, $P=0.93$ for TRPC6; 136±26 versus 100±16 arbitrary units, $P=0.61$ for TRPV1). In contrast, after 24 hours of regular laminar shear stress, the expression of TRPC3 and TRPM7 was significantly higher in HUVECs exposed to shear stress in comparison with HUVECs grown under static conditions (normalized ratio, 374±126 versus 100±19 arbitrary units, $P=0.02$ for TRPC3 and 177±25 versus 100±19 arbitrary units, $P=0.04$ for TRPM7; each $n=5$). Again, the inflammatory properties of the static conditions were demonstrated by the significantly higher expression of TNF- α compared with the dynamic flow profile (normalized ratio, 100±28 versus 16±4 arbitrary units; $P=0.03$; each $n=6$; Figure 4).

To analyze whether the alteration in the expression of TRP channel mRNA expression is PI3K dependent, we repeated the aforementioned experiments with cells that were pre-treated with the PI3K inhibitor LY-294002 and applied a shear stress of 6 dyne/cm² for 4 hours. Even in the presence of LY-294002, laminar shear stress significantly increased TRPC3 in comparison with static conditions (normalized ratio, 423±132 versus 100±13 arbitrary units; $P=0.03$), whereas, in the presence of LY-294002, neither TRPM7 mRNA nor TRPV1 was significantly changed (normalized ratio, TRPM7, 150±74 versus 100±40 arbitrary units; $P=1.0$; TRPV1, 167±50 versus 100±46 arbitrary units; $P=1.0$). TRPC6 mRNA was signifi-

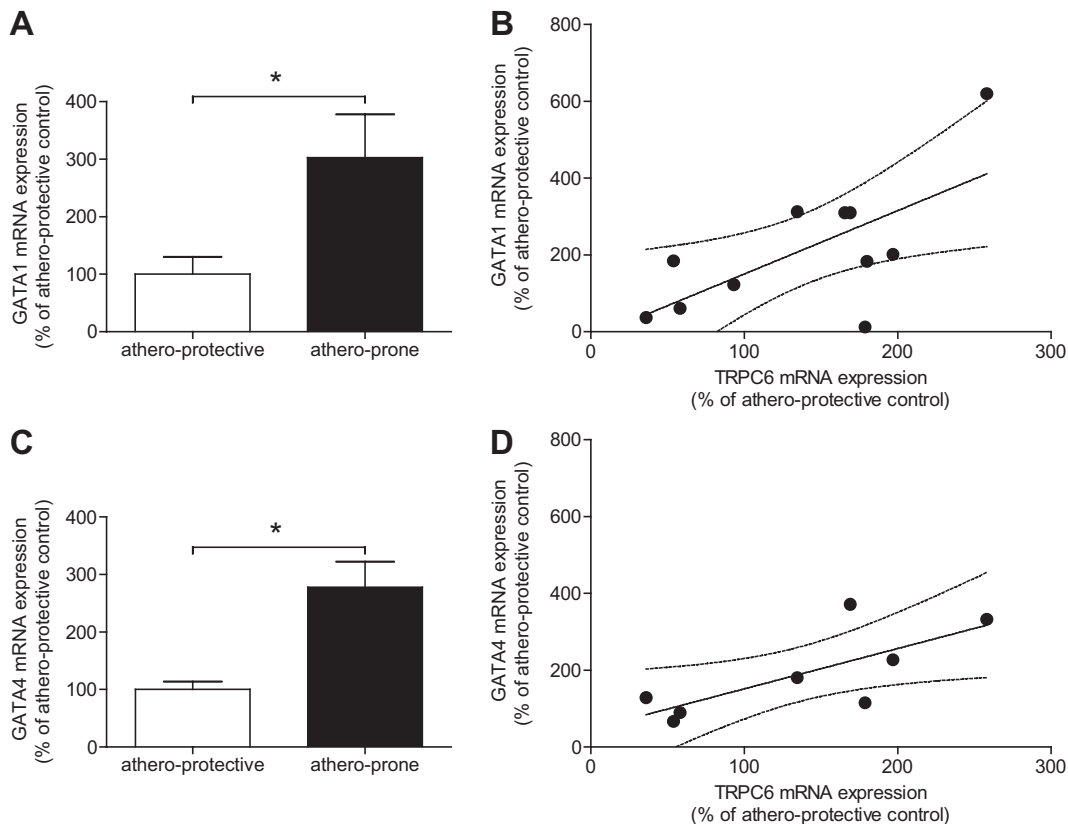


Figure 3. Expression of GATA transcription factors in human umbilical vascular endothelial cells (HUVECs) after flow exposure. HUVECs with medium viscosity increased to 5 cP were exposed to an atheroprone or atheroprotective flow profile for 24 hours, and expression of (A) GATA1 and (C) GATA4 transcripts were analyzed by real-time quantitative RT-PCR. Data show the correlation of GATA1 and GATA4 mRNA expression with TRPC6 mRNA expression in HUVECs after flow exposure. There was a significant correlation between the expression of GATA1 and TRPC6 mRNA ($P=0.02$; B). There was a significant correlation between the expression of GATA4 and TRPC6 mRNA ($P=0.04$; D).

cantly reduced by LY-294002 treatment from 100 ± 10 arbitrary units to 42 ± 9 ($P=0.03$; each $n=6$) in comparison with static conditions. Similar to the experiments without previous inhibition of PI3K, the inflammatory properties of the static conditions were demonstrated by the significantly higher expression of TNF- α compared with the dynamic flow profile (normalized ratio, 100 ± 22 versus 30 ± 6 arbitrary units; $P=0.03$; each $n=6$; Figure 5). These data reveal that the alterations in TRP channel mRNA expression induced by shear stress are not PI3K dependent.

In Silico Prediction of Transcription Factor Binding Sites in the TRPC6 Promoter Region

To indicate possible pathways for the regulation of TRPC6, we analyzed the TRPC6 core promoter and searched for binding sites for GATA transcription factors, which are known to be regulated by shear stress,¹⁸ which are acknowledged to regulate TRPC6 as part of a signaling circuit during pathological cardiac remodeling,¹⁷ and which play a significant role in endothelial cells, for example, in vessel formation, endothelial cell migration, and apoptosis.¹⁹ We, therefore, performed an in silico prediction for GATA transcription factor binding sites and their binding affinities (according to Transcription Element Search System Database) in the core promoter region 2 kb upstream of the ATG start site (chr11: 101454235–101456234; -strand) of the

human TRPC6 gene on chromosome 11 using the Transcription Element Search System (Figure 6).

Expression of TRP Channel mRNA in Atherosclerotic Vessels

Next we analyzed TRPC6 and TNF- α mRNA expressions in atherosclerotic vessels from humans. Small parts of atherosclerotic arteries were obtained from chronic kidney disease patients ($n=6$; age, 56 ± 4 years; 3 men, 3 women; body mass index, 25.4 ± 3.0 kg/m²). In line with the findings in vitro, there was a significant association between the expression of TRPC6 and TNF- α mRNA ($r=0.89$; $P<0.05$) in vascular tissue from chronic kidney disease patients.

Discussion

The present study demonstrates that transcripts of different TRP channels are differentially regulated under various shear stress protocols. Our findings implicate selective TRP gene products as contributors to the vascular remodeling process. We used several well-described shear stress profiles. First, the atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm² was described by Dai et al⁴ in 2004 and was chosen in the present study because it represents atherosclerosis-susceptible regions of human arteries and is seen at atheroprone sites. This atheroprone shear stress waveform is significantly different from the sinusoid wave-

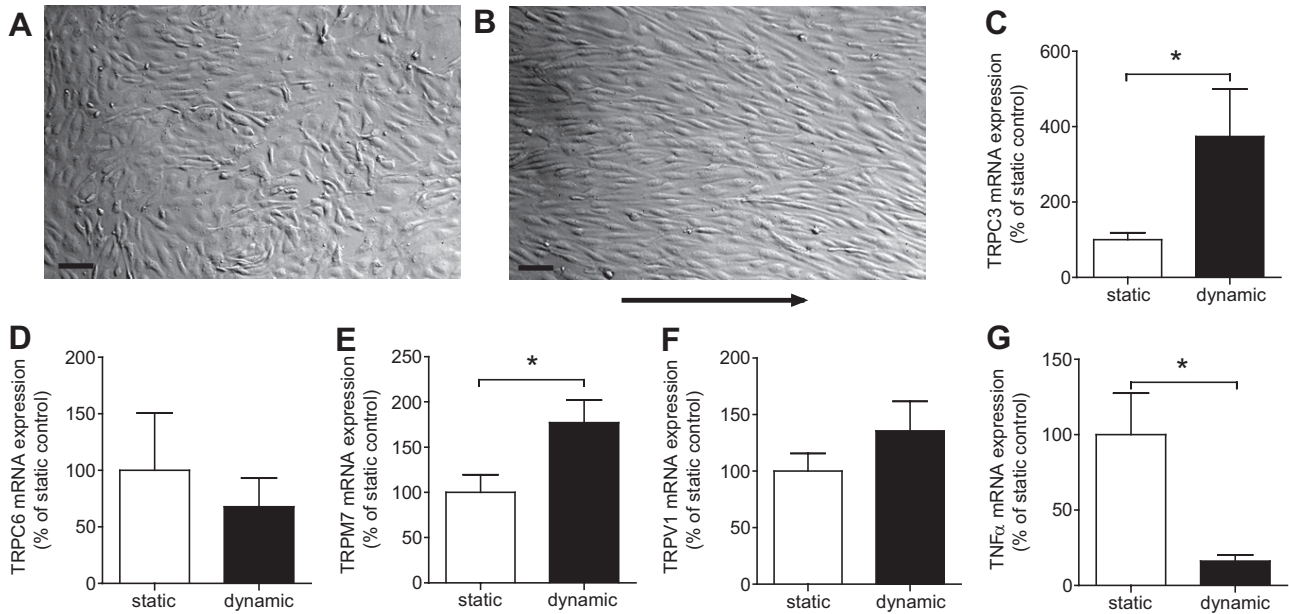


Figure 4. Morphology of human umbilical vascular endothelial cells (HUVECs) after flow exposure. HUVECs were exposed to control (static) conditions (A) or dynamic shear stress (6 dyne/cm², 24 hours; B). There was a significant HUVEC alignment in response to the dynamic shear stress applied compared to control (static) conditions for 24 hours. Scale bars indicate 100 μm. **Arrow** indicates direction of shear stress. Data show changes in mRNA expression by laminar shear stress. HUVECs were exposed to dynamic shear stress (6 dyne/cm², 24 hours) or kept under static control conditions and analyzed for differential expression of mRNA transcripts of (C) TRPC3, (D) TRPC6, (E) TRPM7, (F) TRPV1, and (G) TNF-α by quantitative RT-PCR. **P* < 0.05 between groups.

form, which is also used as a paradigm for arteriosclerosis-susceptible regions.⁴ Second, the atheroprotective flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm² represents regions that are resistant to the development of atherosclerotic lesions. Third, static, that is, no-flow conditions, represent nonperfused

vessels and occur in vivo only in sprouting capillaries and after vessel occlusion.^{16,20–22} Morphological changes represent a late response of endothelial cells on flow exposure.²³ The experiments performed confirmed typical morphological changes of human endothelial cells under the influence of atheroprotective shear stress²⁴ versus atheroprone²⁵ and static

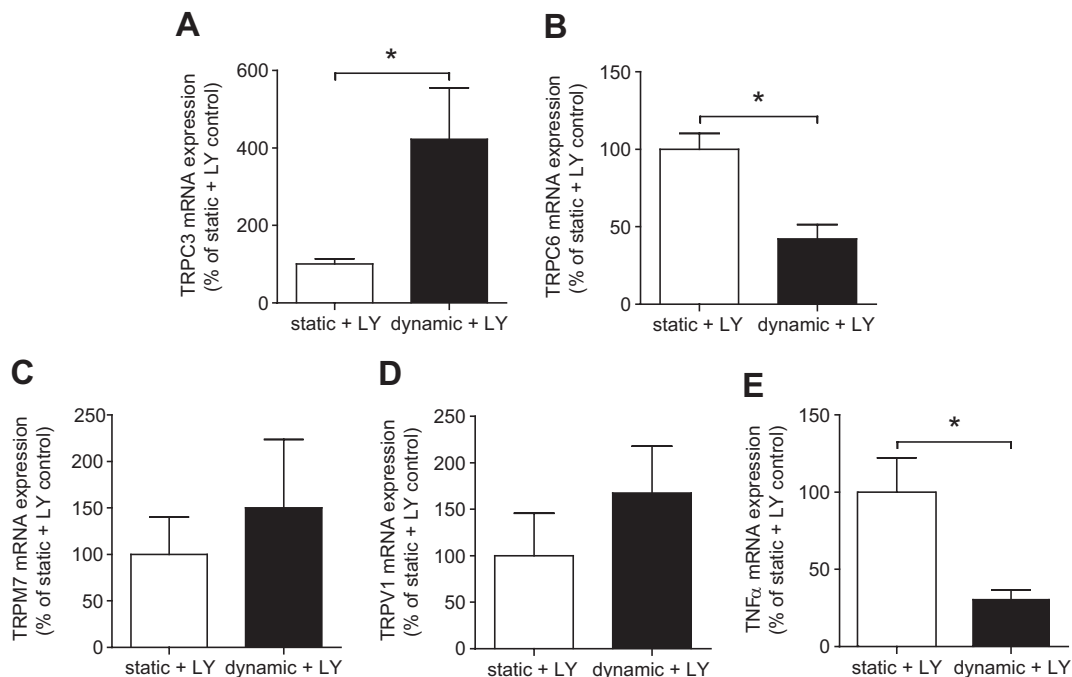


Figure 5. Influence of shear stress and inhibition of phosphatidylinositol 3-kinase (PI3K) on expression of mRNA. Human umbilical vascular endothelial cells (HUVECs) were kept under static conditions, dynamic conditions with shear stress of 6 dyne/cm² for 4 hours, or dynamic conditions with shear stress of 6 dyne/cm² for 4 hours after treatment with LY-294002 (10 μmol/L; +Ly), and expression of (A) TRPC3, (B) TRPC6, (C) TRPM7, (D) TRPV1, and (E) tumor necrosis factor (TNF)-α mRNA was measured by quantitative RT-PCR. **P* < 0.05 between groups.

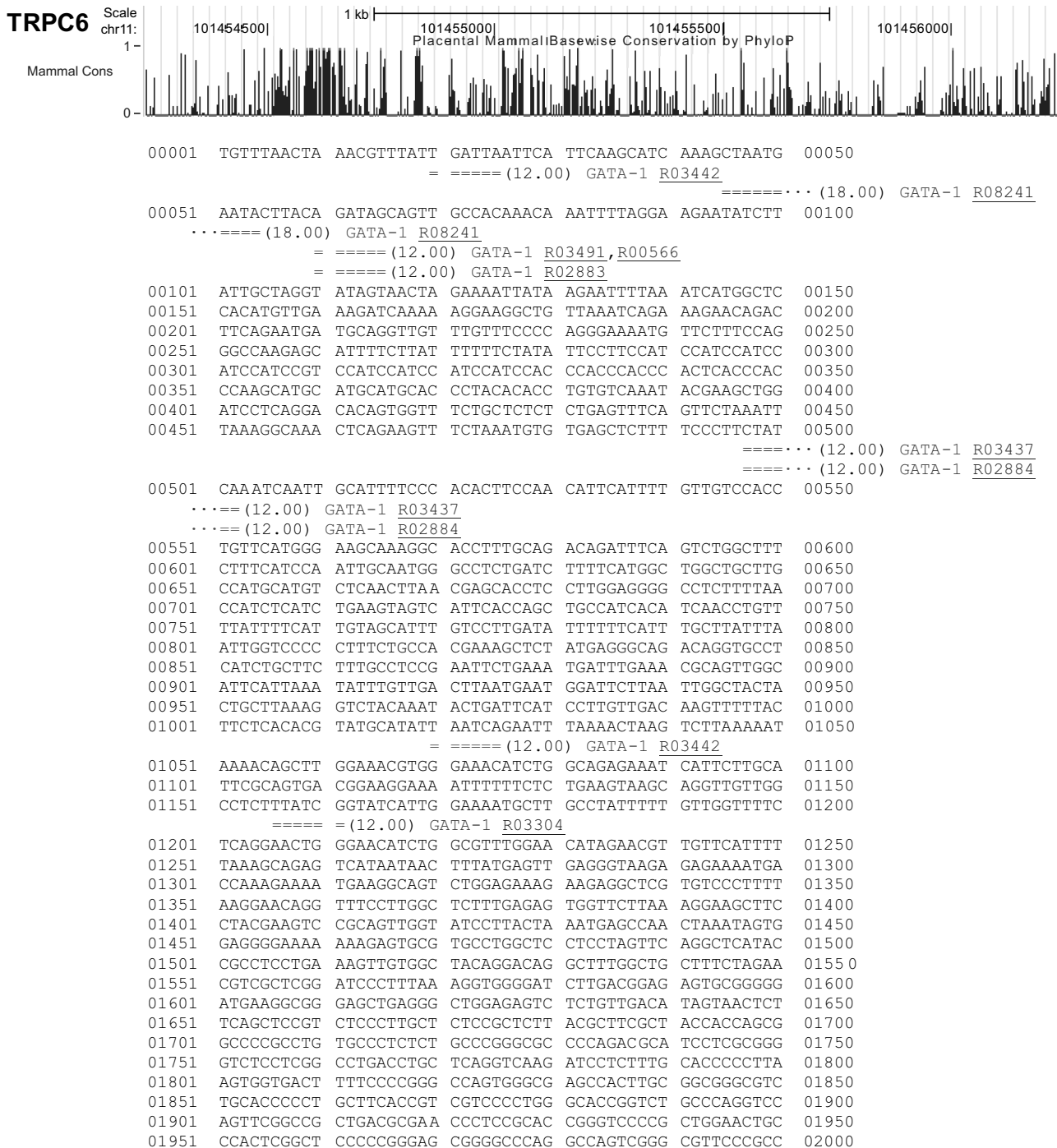


Figure 6. In silico analysis of the human TRPC6 core promoter region, 2 kb upstream of the respective ATG start site (chromosome 11: 101 454 235–101 456 234; -strand). **Track 1**, Chromosomal location and sequence conservation among species (according to PubMed Database PhyloP). **Track 2**, Positions of predicated transcription binding sites for GATA1 and their respective binding affinities (according to Transcription Element Search System Database).

conditions²⁶ versus dynamic shear stress.^{1,27–29} The inflammatory properties of the atheroprone flow profile in comparison with the atheroprotective flow profile were well underlined by the significant higher expression of TNF- α mRNA in the former group in the present study. Finally, regular laminar shear stress of ≤ 6 dyne/cm² was chosen because it can be present at every part of the vascular tree and represents the typical average level in human venous (≈ 1 dyne/cm²) and arterial vessels (≈ 10 dyne/cm²). This profile represents

well-perfused mature vessels.^{20–22} In our study, the expression of TNF- α mRNA, which was used as a marker of inflammation, tended to be higher under static conditions compared with the dynamic flow profile; however, it failed to reach significance probably because of the lower number of experiments.

Specific flow characteristics and the shear stress produced by them are linked to atherosclerosis,^{1,2} for example, in vivo no-flow conditions occur after vessel occlusion (and in

sprouting capillaries), and atheroprone flow profiles are observed at atheroprone sites.¹⁶ We showed that a shear stress profile taken from an atheroprone site increases TRPC6 and TRPV1 mRNA expression in endothelial cells. Although atheroprone shear stress increased TRPC6 and TRPV1 transcripts, protein levels were not significantly affected within 24 hours. These data indicate that only long-term exposure to atheroprone shear stress may cause receptor protein modifications. TRPC6 expression in branched vessels has been reported to be associated with increased atherosclerosis. TRPC6 plays a role in vascular endothelial growth factor-mediated microvessel permeability and thrombin-induced endothelial shape change.^{30,31} In addition, it has been shown recently by our group that increased TRPC6 expression is a hallmark of atherosclerosis.¹⁰

Analysis of the TRPC6 promoter revealed possible binding sites for GATA transcription factors, which are known to be regulated by shear stress,¹⁸ which are acknowledged to regulate TRPC6 as part of a signaling circuit during pathological cardiac remodeling,¹⁷ and which play a significant role in endothelial cells, for example, in vessel formation, endothelial cell migration, and apoptosis.¹⁹ Both, the significant association of GATA1 and GATA4 mRNA expression with TRPC6 mRNA expression and the determination of GATA1 binding sites in the promoter region of TRPC6 point to these transcription factors as the underlying mechanisms, by which TRPC6 expression is enhanced by atheroprone shear stress.

There was a significant association between the expression of TRPC6 and TNF- α mRNA in vascular tissue from chronic kidney disease patients. The linkage between shear stress and inflammatory gene response has been the subject of recent publications concluding that Jun N-terminal protein kinase 2 promotes endothelial cell alignment under flow,³² and mitogen-activated protein kinases play an important role in this regard.³³ Moreover, the correlation between TRPC6 and TNF- α experimentally shown in vitro was, thus, similarly shown in vivo.

Perspectives

For the first time, we showed that atheroprone shear stress especially affects TRPC6 and TRPV1 mRNA expression in endothelial cells. Because TRP channels are known to be associated with atherosclerotic effects, such as inflammation and oxidative stress,^{7–13} these results imply that these selective TRP channel gene products are those that contribute to the atherosclerotic process. In line with these findings, Thilo et al^{10,34} showed recently that TRP channels are regulated by oxidative and antioxidative substances. Increased transplasma membrane calcium influx through TRP channels may aggravate the atherosclerotic processes. Up to now, it is only partly known which TRP channels are in which way linked to atherosclerotic events, including increased proliferation and apoptosis of endothelial cells or smooth muscle cells, vascular remodeling, low-density lipoprotein uptake, and/or lipid synthesis.³⁵ However, recent interventional studies in mice indicated that modulating TRPV1 channel activity may reduce atherosclerosis.³⁶ Hence, the present study may indicate that upregulation of TRPC6 and TRPV1 mRNA in human

endothelial cells contributes to transduction of hemodynamic events including shear stress at “disturbed” flow conditions and may, thus, turn the system balance toward the major cardiovascular diseases.

Sources of Funding

The study was supported by Deutsche Forschungsgemeinschaft and Else-Kröner-Fresenius-Stiftung, Germany.

Disclosures

None.

References

- Hahn C, Schwartz MA. Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol.* 2009;10:53–62.
- Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nat Clin Pract Cardiovasc Med.* 2009;6:16–26.
- Gelfand BD, Meller J, Pryor AW, Kahn M, Bortz PD, Wamhoff BR, Blackman BR. Hemodynamic activation of β -catenin and T-cell-specific transcription factor signaling in vascular endothelium regulates fibronectin expression. *Arterioscler Thromb Vasc Biol.* 2011;31:1625–1633.
- Dai G, Kaazempur-Mofrad MR, Natarajan S, Zhang Y, Vaughn S, Blackman BR, Kamm RD, García-Cardena G, Gimbrone MA Jr. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proc Natl Acad Sci USA.* 2004;101:14871–14876.
- Maroski J, Vorderwülbecke BJ, Fiedorowicz K, Da Silva-Azevedo L, Siegel G, Marki A, Pries AR, Zakrzewicz A. Shear stress increases endothelial hyaluronan synthase 2 and hyaluronan synthesis especially in regard to an atheroprotective flow profile. *Exp Physiol.* 2011;96:977–986.
- Gautam M, Shen Y, Thirkill TL, Douglas GC, Barakat AI. Flow-activated chloride channels in vascular endothelium: shear stress sensitivity, desensitization dynamics, and physiological implications. *J Biol Chem.* 2006;281:36492–36600.
- Inoue R, Jensen LJ, Shi J, Morita H, Nishida M, Honda A, Ito Y. Transient receptor potential channels in cardiovascular function and disease. *Circ Res.* 2006;99:119–131.
- Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential channels in disease. *Physiol Rev.* 2007;87:165–217.
- Smedlund K, Vazquez G. Involvement of native TRPC3 proteins in ATP-dependent expression of VCAM-1 and monocyte adherence in coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol.* 2008;28:2049–2055.
- Wuensch T, Thilo F, Krueger K, Scholze A, Ristow M, Tepel M. High glucose-induced oxidative stress increases transient receptor potential channel expression in human monocytes. *Diabetes.* 2010;59:844–849.
- Liu Y, Xu Y, Thilo F, Friis UG, Jensen BL, Scholze A, Zheng J, Tepel M. Erythropoietin increases expression and function of transient receptor potential canonical 5 channels. *Hypertension.* 2011;58:317–324.
- Yogi A, Callera GE, Antunes TT, Tostes RC, Touyz RM. Transient receptor potential melastatin 7 (TRPM7) cation channels, magnesium and the vascular system in hypertension. *Circ J.* 2011;75:237–245.
- Tanaka T, Ikeda K, Yamamoto Y, Iida H, Kikuchi H, Morita T, Yamasoba T, Nagai R, Nakajima T. Effects of serum amyloid A and lysophosphatidylcholine on intracellular calcium concentration in human coronary artery smooth muscle cells. *Int Heart J.* 2011;52:185–193.
- Bongrazio M, Pries AR, Zakrzewicz A. The endothelium as physiological source of properdin: role of wall shear stress. *Mol Immunol.* 2003;39:669–675.
- Bongrazio M, Baumann C, Zakrzewicz A, Pries AR, Gaetgens P. Evidence for modulation of genes involved in vascular adaptation by prolonged exposure of endothelial cells to shear stress. *Cardiovasc Res.* 2000;47:384–393.
- Hohberg M, Knöchel J, Hoffmann CJ, Chlench S, Wunderlich W, Alter A, Maroski J, Vorderwülbecke BJ, Da Silva-Azevedo L, Knudsen R, Lehmann R, Fiedorowicz K, Bongrazio M, Nitsche B, Hoepfner M, Styp-Rekowska B, Pries AR, Zakrzewicz A. Expression of ADAMTS1 in endothelial cells is induced by shear stress and suppressed in sprouting capillaries. *J Cell Physiol.* 2011;226:350–361.

17. Kuwahara K, Wang Y, McAnally J, Richardson JA, Bassel-Duby R, Hill JA, Olson EN. TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. *J Clin Invest*. 2006;116:3114–3126.
18. Sokabe T, Yamamoto K, Ohura N, Nakatsuka H, Qin K, Obi S, Kamiya A, Ando J. Differential regulation of urokinase-type plasminogen activator expression by fluid shear stress in human coronary artery endothelial cells. *Am J Physiol Heart Circ Physiol*. 2004;287:H2027–H2034.
19. Fan C, Ouyang P, Timur AA, He P, You SA, Hu Y, Ke T, Driscoll DJ, Chen Q, Wang QK. Novel roles of GATA1 in regulation of angiogenic factor AGGF1 and endothelial cell function. *J Biol Chem*. 2009;284:23331–23343.
20. Langille BL. Remodeling of developing and mature arteries: endothelium, smooth muscle, and matrix. *J Cardiovasc Pharmacol*. 1993;21:S11–17.
21. Pries AR, Secomb TW, Gaehtgens P. Design principles of vascular beds. *Circ Res*. 1995;77:1017–1023.
22. Chlench S, Mecha Disassa N, Hohberg M, Hoffmann C, Pohlkamp T, Beyer G, Bongrazio M, Da Silva-Azevedo L, Baum O, Pries AR, Zakrzewicz A. Regulation of Foxo-1 and the angiotensin-2/Tie2 system by shear stress. *FEBS Lett*. 2007;581:673–680.
23. Rouleau L, Farcas M, Tardif JC, Mongrain R, Leask RL. Endothelial cell morphologic response to asymmetric stenosis hemodynamics: effects of spatial wall shear stress gradients. *J Biomech Eng*. 2010;132:081013.
24. Hastings NE, Simmers MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. *Am J Physiol Cell Physiol*. 2007;293:C1824–C1833.
25. Conway DE, Williams MR, Eskin SG, McIntire LV. Endothelial cell responses to atheroprone flow are driven by two separate flow components: low time-average shear stress and fluid flow reversal. *Am J Physiol Heart Circ Physiol*. 2010;298:H367–H374.
26. Inoguchi H, Tanaka T, Maehara Y, Matsuda T. The effect of gradually graded shear stress on the morphological integrity of a huvec-seeded compliant small-diameter vascular graft. *Biomaterials*. 2007;28:486–495.
27. Miyazaki T, Honda K, Ohata H. Requirement of Ca²⁺ influx- and phosphatidylinositol 3-kinase-mediated m-calpain activity for shear stress-induced endothelial cell polarity. *Am J Physiol Cell Physiol*. 2007;293:C1216–C1225.
28. Ives CL, Eskin SG, McIntire LV. Mechanical effects on endothelial cell morphology: in vitro assessment. *In Vitro Cell Dev Biol*. 1986;22:500–507.
29. Nerem RM, Girard PR. Hemodynamic influences on vascular endothelial biology. *Toxicol Pathol*. 1990;18:572–582.
30. Pocock TM, Foster RR, Bates DO. Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. *Am J Physiol Heart Circ Physiol*. 2004;286:H1015–H1026.
31. Singh I, Knezevic N, Ahmed GU, Kini V, Malik AB, Mehta D. Gαq-TRPC6-mediated Ca²⁺ entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. *J Biol Chem*. 2007;282:7833–7843.
32. Hahn C, Wang C, Orr AW, Coon BG, Schwartz MA. JNK2 promotes endothelial cell alignment under flow. *PLoS One*. 2011;6:e24338.
33. Yoshizumi M, Abe J, Tsuchiya K, Berk BC, Tamaki T. Stress and vascular responses: atheroprotective effect of laminar fluid shear stress in endothelial cells: possible role of mitogen-activated protein kinases. *J Pharmacol Sci*. 2003;91:172–176.
34. Thilo F, Liu Y, Krueger K, Foerste N, Wittstock A, Scholze A, Tepel M. Do cysteine residues regulate transient receptor potential canonical type 6 (TRPC6) channel protein expression? *Antioxid Redox Signal*. 2012;16:452–457.
35. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev*. 2011;91:327–387.
36. Ma L, Zhong J, Zhao Z, Luo Z, Ma S, Sun J, He H, Zhu T, Liu D, Zhu Z, Tepel M. Activation of TRPV1 reduces vascular lipid accumulation and attenuates atherosclerosis. *Cardiovasc Res*. 2011;92:504–513.

Pulsatile Atheroprone Shear Stress Affects the Expression of Transient Receptor Potential Channels in Human Endothelial Cells

Florian Thilo, Bernd J. Vorderwülbecke, Alex Marki, Katharina Krueger, Ying Liu, Daniel Baumunk, Andreas Zakrzewicz and Martin Tepel

Hypertension. 2012;59:1232-1240; originally published online May 7, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.183608

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://hyper.ahajournals.org/content/59/6/1232>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Hypertension* is online at:
<http://hyper.ahajournals.org/subscriptions/>